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Tributyltin (TBT) Stimulates Synthesis of Interferon gamma (IFNγ) and Tumor Necrosis Factor Alpha (TNFα) in Human Lymphocytes

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Abstract

Tributyltin (TBT) is found in human blood and other tissues and thus is of considerable concern as to its effects on human health. Previous studies have demonstrated that TBT has detrimental effects on immune function. Recently, we found that exposures to TBT caused increased secretion of two important pro-inflammatory cytokines, tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ). Elevation of either of these cytokines has the potential to cause chronic inflammation, which is an important factor in a number of diseases including cancer. The current study examined the mechanism of TBT-induced elevations of TNFα and IFNγ secretion and found that the p38 MAPK pathway was essential to the ability of TBT to stimulate secretion. Additionally, this study demonstrated that increased secretion of these cytokines was due to TBT-induced increases in their overall synthesis, rather than simply being due to an increase in the release of already formed proteins. The TBT-induced increases in synthesis were evident within 6 h of exposure. The p38 MAPK pathway is also necessary for the TBT-induced increases in both TNFα and IFNγ synthesis. The role of increased transcription of TNFα and IFNγ mRNA in response to TBT exposures as a possible explanation for the increased synthesis of these cytokines was also examined. It was found that increased mRNA levels did not appear to fully explain the increases in either TNFα or IFNγ synthesis. Thus, TBT is able to increase secretion of two important pro-inflammatory cytokines by increasing their synthesis.

INTRODUCTION

Tributyltin (TBT) has been used as an ingredient in antifouling paints as well as an antifungal agent and biocide in textiles, paper production, industrial cooling waters and wood preservation (Kimbrough, 1976; Laughlin and Linden, 1985; Roper, 1992). Although TBT uses have been banned or severely restricted in many parts of the world, due to its persistent properties, residues of TBT are still found in the environment and cause harmful health effects in wildlife and humans (Gipperth, 2009; Loganathan *et al.*, 2000; Loganathan, 2016, Kirchner *et al.*, 2010; Gao *et al.* 2017). Human exposures to TBT are thought to mainly arise from the intake of contaminated food (WHO, 1990; Kannan *et al.*, 1995). Blood

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levels of TBT range as high as 261 nM (85 ng/mL) (Whalen et al., 1999; Kannan *et al.*, 1999). Other sources for dermal or pulmonary exposures include, disinfectants in specific wax, polish and cleansing products, catalysts and pesticides (Kannan *et al.*, 1999; Takahashi *et al.*, 1999). The exposure of mammals to TBT has been shown to cause increased incidences of tumors (Wester *et al.*, 1990), decreased NK cell function (Ghoneum *et al.*, 1990), thymic atrophy and thymus dependent immunosuppression (Snoeij *et al.*, 1987, 1989; Vos *et al.* 1990). TBT has also been detected in organs such as the heart, liver, kidney and stomach (Gui-Bin *et al.*, 2000). TBT decreases lytic function, target-binding function, cell-surface protein expression and cytolytic protein expression in human natural killer (NK) cells at levels that are in the range found in human blood (Whalen *et al.*, 1999; Dudimah *et al.*, 2007; Whalen *et al.*, 2002; Thomas *et al.*, 2004).

Recent studies have shown that exposure to TBT alters the secretion of several pro-inflammatory cytokines including, interleukin 1 beta (IL-1β), interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα), and IL-6 from human immune cells (Hurt *et al.* 2013; Lawrence *et al.*, 2015; Brown and Whalen, 2015; Brown *et al.*, 2017).

IFNγ is produced by T cells, NK cells, and to a more limited extent by myeloid lineage cells such as monocytes and macrophages (Billiau and Matthys, 2009; Darwich *et al.*, 2008; Andoniou *et al.*, 2008; Girart *et al.*, 2007). TNFα is made by various cell types including, lymphocytes, monocytes, macrophages, smooth muscle cells, fibroblasts, endothelial cells, epithelial cells, adipocytes and osteoblasts with activated macrophages and T lymphocytes being the predominant producers of TNFα (Locksley, 2001). Elevated levels of both IFNγ and TNFα are associated with chronic inflammation which has been linked to the development of a number of diseases including cancer (Macarthur *et al.*, 2004; Balkwill and Mantovani, 2001). Elevated levels of IFN have been shown to facilitate the development of gastrointestinal cancers (Macarthur *et al.*, 2004) and elevated TNFα may alter the proliferation and invasiveness of tumor cells, due to its capacity to act as a growth factor, angiogenic factor and inducer of epithelial-mesenchymal transition (Vajdic and van Leeuven, 2009). The secretion and synthesis of both IFNγ and TNFα are regulated by mitogen activated protein kinases (MAPKs) (Schoenborn and Wilson, 2007; Gaestel *et al.*, 2009) and by nuclear factor kappa B (NFκB) (Strengell *et al.*, 2003; Gaestel *et al.*, 2009). Additionally, TNFα converting enzyme, TACE, is needed for the secretion of the soluble form of TNFα (Goetz *et al.*, 2004).

Tight regulation of levels of both IFNγ and TNFα are necessary to prevent loss of immune competency (too little) or chronic inflammation (too much). As mentioned above, TBT has been shown to dysregulate the secretion of both TNFα and IFNγ from immune cells (Hurt *et al.* 2013; Lawrence *et al.*, 2015).

In the current study, we examine the signaling pathways that may regulate the previously described TBT-induced increases in secretion of these two important pro-inflammatory cytokines (Hurt *et al.* 2013; Lawrence *et al.*, 2015). Additionally, studies are carried out to determine whether TBT-induced alterations in the synthesis of IFNγ and/or TNFα are occurring. It is possible that TBT only alters the secretory pathway of already existing cytokines, without affecting de novo synthesis of these proteins. Finally, IFNγ and TNFα

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mRNA levels are examined to determine if there are TBT-induced changes in the levels of these transcripts that are responsible for any alterations in protein synthesis.

MATERIALS AND METHODS

Preparation of monocyte- depleted PBMCs

PBMCs were isolated from Leukocyte filters (PALL- RC2D) obtained from the Red Cross Blood Bank Facility (Nashville, TN) as described in Meyer *et al.*, 2005. Leukocytes were retrieved from the filters by back-flushing them with elution medium (PBS containing 5 mM disodium EDTA and 2.5% [w/v] sucrose) and collecting the eluent. Eluent was layered onto Ficoll-Hypaque (1.077g/mL) and centrifuged at 1200 g for 30 min. PBMCs were collected and washed (250 g, 10 min) with PBS. Cells were then suspended in complete medium which consisted of RPMI-1640 supplemented with 10% heat-inactivated BCS, 2 mM L-glutamine and 50 U penicillin G with 50 μg streptomycin/mL. Monocyte-depleted PBMCs (10–20% $CD16^+$, 10–20 % $CD56^+$, 70–80% $CD3^+$, 3–5% $CD19^+$, 2–20% $CD14^+$) were prepared by incubating the cells in glass Petri dishes (150 × 15 mm) at 37 °C and air/CO_2, 19:1 for a total of 1.5 h.

Chemical preparation

TBT was purchased from Sigma-Aldrich (St. Louis, MO). TBT was a neat standard, dissolved initially in deionized water to give a 1 mM solution. Desired concentrations of TBT were prepared by dilution of the stock into complete media.

Cell treatments

MD-PBMCs were treated with TBT with appropriate control at concentrations of 2.5–200 nM for 10 min, 30 min, 6 h, and 24 h. Following the incubations, the cells were pelleted and the supernatants were collected and frozen at -70°C until assay. Cell pellets were lysed and stored at −70°C for western blot analysis.

Inhibitor Preparation

Enzyme inhibitors were purchased from Fischer Scientific (Pittsburgh, PA). The stock solution for each inhibitor was a 50 mM solution in dimethylsulfoxide (DMSO). JNK Inhibitor (BI78D3), MEK1/2 pathway inhibitor (PD98059), p38 inhibitor (SB202190), NFκB inhibitor (BAY11-7085) and TACE inhibitor (Batimastat) were prepared by dilution of the stock solution into cell culture media.

Cell treatments with Inhibitors

For pathway inhibitor experiments, MD-PBMCs were treated with pathway inhibitors 1h before adding TBT at concentrations of 5, 10, 25 nM TBT for 24 h. Following the incubations, the cells were pelleted and supernatants were collected and stored at −70 °C until assaying for IFNγ and TNFα.

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Cell viability

Cell viability was assessed at the beginning and end of each exposure period. Viability was determined using the trypan blue exclusion method. Cells were mixed with trypan blue and counted using a hemocytometer. The total number of cells and the total number of live cells were determined for both control and treated cells to determine the percent viable cells. Viability was not significantly affected with any of the treatments.

IFNγ and TNFα secretion assay

IFNγ and TNFα levels were assessed using the OptEIA™ enzyme-linked immunosorbent assay (ELISA) human IFNγ and TNFα kits (BD-Pharmingen, San Diego, CA) respectively. Briefly, appropriate capture antibody was applied to the wells of a 96 well flat-bottom microwell plate specifically designed for ELISA (Fisher, St. Louis MO) after removal of excess capture antibody (by washing with PBS containing 0.05% Tween-20), the wells were treated with blocking buffer. Blocking buffer was removed and cell supernatants and IFNγ or TNFα standards were added to the plate. Following the incubation with samples and standards, detection antibody was added. Following the removal of the detection antibody, a substrate solution was added to each well. Incubation with substrate was ended by addition of acid and the absorbance measured at 450 nm on a Thermo Labsystems Multiskan MCC/340 plate reader (Fisher Scientific).

Gel Electrophoresis and Western Blotting

MD-PBMCs at a concentration of 4 million/0.67mL were exposed to TBT for 10 min, 30 min, 6 h or 24 h. Following the treatments, the cells were centrifuged and the cell pellets lysed using 133μL of lysis buffer (Active motif, Carlsbad, CA) 4 million cells. Cell lysates were then stored frozen at −80 °C until they were run on 10% SDS-PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis). Control and treated cells for a given experimental setup were from an individual donor. The PVDF membrane was immunoblotted with anti-IFNγ, anti-TNFα and anti- β-actin antibodies (Cell signaling Technologies, Beverly, MA). Antibodies were then visualized using an ECL chemiluminescent detection system (Amersham, Piscataway, NJ) and UVP Software. The density of each protein band was determined by densitometric analysis using the UVP analysis software. The settings on the image station were optimized to detect the largest possible signal range and prevent saturation of the system. Differences in protein expression were determined relative to an internal control. This determination provided comparative quantitation by evaluating whether a given treatment changed the expression of IFNγ and TNFα relative to untreated cells. β-actin levels were determined for each condition to verify that equal amounts of protein were loaded. Additionally, the density of each protein band was normalized to β- actin to correct for any minor differences in the loading among the lanes.

RNA Isolation and RT-qPCR

RNA from MD-PBMCs was extracted with RNeasy Mini kit (Qiagen). RNA concentrations were measured with a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). PCR primers were designed using Primer Express 2.0 (Applied Biosystems):

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IFNγ forward sequence	TGC AAT CTG AGC CAG TGC TT
IFNγ reverse sequence	CAG GGT CAC CTG ACA CAT TCA A
TNFα forward sequence	TCC CCT GCC CCA ATC C
TNFα reverse sequence	CCA ATT CTC TTT TTG AGC CAG AA

All RT-qPCR assays were conducted using QuantiTect SYBR Green RT-PCR kit (Qiagen). Reaction was done in 20 μL containing 50 ng of total RNA and 0.4 μM of each primer. Thermal cycles contained one cycle of pre-incubation at 50 °C for 10 minutes and 95 °C for 15 minutes, 35 cycles of amplification (95 °C for 15 seconds and 60 °C for 60 seconds). Primers were validated by melting curve analysis, standard curve and non-template control reactions. Each concentration was analyzed in duplication with RT- qPCR to determine amplification efficiency.

Statistical Analysis

Statistical analysis of the data was performed by using ANOVA and Student's t test. Data were initially compared within a given experimental setup by ANOVA. A significant ANOVA was followed by pair wise analysis of control versus exposed data using Student's t test, a p value of less than 0.05 was considered significant.

RESULTS

Effects of TBT Exposures on IFNγ Secretion from MD-PBMCS Treated with Selective Enzyme Inhibitors

NFκB inhibitor (BAY 11-7085)—MD-PBMCs were exposed to the NFκB inhibitor (BAY 11-7085) for 1 h, before a 24 h TBT exposure to 50, 25 and 10 nM TBT. These concentrations of TBT were examined as one or more had produced significant elevations in IFNγ secretion in previous studies (Lawrence *et al.*, 2015). Cells from 4 different donors all showed increased secretion of IFNγ after exposures to 50, 25, or 10 nM TBT. When the NFκB pathway was inhibited, all donors showed somewhat diminished TBT-induced IFNγ secretion. For instance, cells from donor F380 showed increases of 2.9, 3,8 and 5.2 fold with exposures to 10, 25 and 50 nM TBT. When the inhibitor was present, these same concentrations of TBT showed diminished effects on IFNγ secretion of 1.6, 3.0 and 3.7 fold (Figure 1A). Results were replicated in cells prepared from 3 additional donors. These results indicate that the NFκB pathway is needed to a limited extent for TBT-induced secretion of IFNγ.

JNK inhibitor (B178D3)—When the JNK pathway was inhibited by a 1 h pre-treatment with JNK inhibitor (B178D3), the ability of TBT to stimulate increased secretion of IFNγ from lymphocytes was unchanged. This effect was seen in MD-PBMCs from 4 individual donors. The resultsindicated that TBT-induced secretion of IFNγ was not dependent on activation of the JNK pathway (data not shown). Thus the JNK pathway cannot be seen as essential to TBT-induced increases in IFNγ.

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Mitogen activated protein kinase kinase (MAP2K)/ MEK Inhibitor (PD98059)— When the ERK1/2 pathway was inhibited prior to exposing cells to TBT, there was no consistent effect on TBT-induced increases in IFNγ (data not shown). These results suggest that TBT does not require the ERK1/2 pathway to induce increase in IFNγ secretion.

p38 Inhibitor (SB202190)—Blocking the p38 pathway decreased the ability of TBT to induce elevations of IFNγ in cells from 4 separate donors. Results indicated that the p38 pathway is needed for TBT to elevate IFNγ. For instance, cells from donor F380 showed increases of 2.4, 2.4 and 1.5 fold with exposures to 10, 25, and 50 nM TBT. After inhibition of the p38 pathway, these same concentrations showed a dramatic reduction in TBT-induced IFNγ secretion (Figure 1B). Similar results were seen in all donors tested. These data suggest that TBT heavily relies on the p38 pathway to promote increases in IFNγ secretion.

Effects of TBT exposures on cellular synthesis of IFNγ (secretion + intracellular levels)

10 min exposure to TBT—Both secreted and intracellular levels of IFNγ were measured in cells from the same donor. After exposure to TBT (0–200 nM) the supernatants were collected for measuring secreted IFNγ and the cells were lysed to measure intracellular levels. Synthesis of IFNγ is measured as the combined effect on intracellular levels and secreted levels. Cells from a total of 4 individual donors were examined for the effects of TBT on IFNγ secretion. There was no consistent effect of TBT exposures on IFNγ production seen after 10 minutes (data not shown).

30 min exposure to TBT—The synthesis of IFNγ in MD-PBMCs exposed to 0–200 nM TBT for 30 minutes were analyzed from 4 separate donors (data not shown). Again, there was no consistent effect of TBT exposures on the synthesis of IFNγ after 30 min of exposure.

6 h exposure to TBT—Synthesis (intracellular + secreted levels) of IFNγ after exposure to TBT for 6 h was increased at 1or more concentration of TBT in cells from each of the 4 donors that were examined (Figure 2A). For instance, cells from donor F234 showed increased synthesis (secreted+intracellular levels) of IFNγ at each TBT exposure. The increases ranged from 1.1 fold at the 200 nM TBT concentration to 2.3 fold at the 2.5 nM exposure. The extent of the increases in synthesis and the concentrations at which they occurred varied among the donors. The fold increase compared to control for cells from 3 other donors are summarized in the bar chart of Figure 2A (control level =1). The results indicate that TBT-induced increases in IFNγ are detectable within 6 h of exposure to TBT.

24 h exposure to TBT—The results of 24 h exposure of MD-PBMCs to 200–2.5nM TBT on the synthesis of IFNγ from 4 donors are shown in Figure 2B. After 24h of exposure to TBT, cells from all donors demonstrated increased synthesis of IFNγ at a minimum of 4 concentrations. For example, cells from donor F200 showed significant increases in synthesis at 200, 100, 50, 25, 10 and 2.5 nM TBT. Fold increases in synthesis for cells from donor F200 (fold increases in intracellular and secreted levels combined) at each of these concentrations were 25, 5.6, 3.3, 2.5, 2.8, and 1.5, respectively. The fold increases in synthesis of IFNγ stimulated by TBT in cells from the other donors are shown in the bar

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chart of Figure 2B. These data indicate that very substantial changes in IFNγ synthesis occur after an incubation of 24 h with TBT.

Effect of p38 pathway inhibitor (SB202190) on TBT-induced increases in IFNγ synthesis

As p38 was shown to be essential for TBT-induced increases in IFNγ secretion, the effects of the p38 inhibitor, SB202190, were examined on TBT-induced increases in IFNγ synthesis. Results from pre-treating MD-PBMCs with SB202190 for 1 h prior to exposing to 10, 25, and 50 nM TBT for 24 h are shown in Figure 2C (representative experiment). Cells from donor F443 showed TBT-induced increases in TNFα synthesis of 1.8 at 50 nM and 1.9 at 25 nM and these were decreased to 1.2 (50 nM) and no increase (25 nM) when the p38 pathway was inhibited. Similar results were seen in cells from 3 additional donors (data not shown). These data indicate that like TBT-induced increases in IFNγ secretion, TBT requires the p38 MAPK pathway to induce increases in IFNγ production.

Effects of 6 h and 24 h exposures to TBT on IFNγ mRNA levels in MD-PBMCs

RT-qPCR was carried out to examine whether TBT-induced changes in IFNγ synthesis were due to changes in the levels of mRNA. MD-PBMCs from 4 donors were exposed to 0–200 nM TBT for 6 h and 24 h (Table 1). mRNA levels were increased in cells from 1 of 4 donors tested after 6 h of incubation and in cells from 2 of 4 donors after 24 h of incubation. Increased mRNA was seen in cells from half of the donors tested by 24 h. Thus, it appears that TBT may at least in some instances be increasing synthesis of IFNγ by increasing in IFNγ mRNA.

Effects of TBT Exposures on TNFα Secretion from MD- PBMCS Treated with Selective Enzyme Inhibitors

A previous study from our lab showed that TNFα secretion was elevated by exposures to 10, 25, and 50 nM TBT (Hurt *et al.*, 2013). Here the mechanism by which TBT induces elevations in TNFα secretion was investigated using inhibitors of signaling pathways, known to be utilized in the production of TNFα as described in the introduction.

NFκB Inhibitor (BAY 11–7085)—When the NFκB pathway was inhibited, there was no consistent change in the TBT-induced increases in TNFα secretion (data not shown). These results indicated that the NFκB pathway is not essential for TBT-induced secretion of TNFα.

JNK Inhibitor (BI78D3)—MD-PBMCs pre-treated with the JNK pathway inhibitor (B178D3) still exhibited TBT-induced increases in TNFα when exposed to 50, 25, and 10 μM TBT (data not shown). These results suggest that the JNK pathway is not needed for TBT-induced increases in TNFα secretion.

Mitogen activated protein kinase kinase (MAP2K)/ MEK Inhibitor (PD98059)—After inhibition of the ERK1/2 pathway, TBT was able to induce increased secretion of TNFα from cells from all donors examined (data not shown). This indicates that TBT is not utilizing this pathway to stimulate TNFα secretion.

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p38 Inhibitor (SB202190)—Inhibiting the p38 pathway with SB202190 decreased TBT-induced increases in TNFα secretion in cells prepared from 4 different donors. These data indicated that the p38 pathway is needed for TBT to elevate TNFα. Cells from a representative donor (F370) showed increases of 1.5, 1.8 and 2.1 fold with exposures to 10, 25, and 50 nM TBT (Figure 3A). After inhibition of the p38 pathway, these same concentrations showed no TBT-induced increase in TNFα secretion. Results were replicated in cells prepared from 3 additional donors (data not shown).

TACE Inhibitor—Inhibiting TACE with batimastat also decreased the ability of TBT to induce increases in TNFα secretion. When TACE was inhibited cells from all donors showed a diminished response to at least one concentration of TBT. Cells from 4 distinct donors were examined. For instance, cells from donor F274 (Figure 3B) showed increases of 1.5, 3.4 and 5.8 fold following exposures to 10, 25, and 50 nM TBT. After inhibition of TACE, 10 nM TBT caused no increase in TNFα secretion while 25 and 50 nM TBT produced diminished increases of 2.2 and 3.4 fold, respectively. Similar results were seen in cells from 3 other donors (data not shown). These data indicate that TBT requires the activation of TACE to cause increases in TNFα secretion. This reflects the essential role of TACE in the secretion of TNFα.

Effects of TBT exposures on cellular synthesis of TNFα (secretion + intracellular levels)

10 min exposure to TBT—MD-PBMCs exposed to 200–2.5nM TBT for 10 min showed no consistent increases in synthesis of TNFα. Cells from 4 donors were examined (data not shown).

30 min exposure to TBT—As was seen after 10 min, there is no consistent effect of TBT exposures on TNFα synthesis after 30 min, in cells prepared from 4 separate donors (data not shown)

6 h exposure to TBT—Effects of TBT on the synthesis of TNFα are shown in Figure 4A. All donors showed increased TNFα synthesis at 2 or more TBT exposures. Cells from donor F250 showed increased synthesis (secreted+intracellular levels) of TNFα compared to control cells at 3 TBT exposures. (Figure 4A). The fold increase compared to control for 3 other donors are summarized in the bar chart of Figure 4A. These data indicate that increases in TNFα synthesis induced by TBT-exposures are seen in cells from all donors within 6 h of exposure.

24 h exposure to TBT

After 24 h of exposure to TBT, cells from all donors showed increased TNFα synthesis at every concentration of TBT examined (Figure 4B). Thus, while the increased production of TNFα appears consistently after 6 h exposure, it becomes much greater and is seen at many more TBT-exposure levels after 24 h. For example, cells from donor F200 showed significant increases in synthesis (either secretion and/or intracellular levels) at 200, 100, 50, 25, 10, 5, and 2.5 nM TBT. Fold increases in synthesis for cells from F200 (fold increases in intracellular and secreted levels combined), at each of these concentrations were 13.4, 4.9, 3.1, 2.4, 2.2, 1.3, and 2.3, respectively. All other donors showed similar trends for increased

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synthesis of TNFα after 24 h. The fold increase compared to control for the other donors are summarized in the bar chart of Figure 4B.

Effect of p38 pathway inhibitor (SB202190) on TBT-induced increases in TNFα synthesis

p38 Inhibitor (SB202190)—Results from pre-treating MD-PBMCs with SB202190 for 1 h prior to exposing to 10, 25, and 50 nM TBT for 24 h are shown Figure 4C (representative experiment). Cells from all donors showed increased TNFα synthesis in response to TBT and these increases were diminished when the p38 MAPK pathway was inhibited. The cells from donor F433 (Figure 4C) showed TBT-stimulated fold increases in TNFα synthesis of 1.45 at 50 nM and 1.74 at 25 nM and these were completely blocked in the presence of SB202190. Similar results were seen in cells from three additional donors. These data indicate that the increased production of TNFα that occurs in response to TBT exposures requires the p38 pathway.

Effects of 6 h and 24 h exposures to TBT on TNFα mRNA levels in MD-PBMCs

RT-qPCR was used to examine whether TBT-induced changes in TNFα synthesis were due to changes in the levels of mRNA. MD-PBMCs from 4 donors were exposed to 0–200 nM TBT for 6 h or 24 h. The results are shown in Table 2. mRNA levels were increased in cells from 2 of 4 donors tested after 6 h of incubation and in cells from 1 of 4 donors after 24 h of incubation. Increased mRNA was seen in cells from half of the donors tested by 6 h. Thus, it appears that, as with IFNγ, TBT may in some instances be increasing synthesis of TNFα by increasing in TNFα mRNA.

DISCUSSION

IFNγ and TNFα are important regulators of immune responsiveness that are produced primarily by T and NK lymphocytes and to some extent by myeloid cells (Zaidi and Merlino, 2011, Billiau and Matthys, 2009; Darwich *et al.*, 2008; Goetz *et al.*, 2004). It is important to determine the effects of environmental contaminants such as the TBT (which has been found in human blood) (Whalen *et al.*, 1999; Kannan *et al.*, 1999) on the secretion of these potent regulators. TBT-induced alterations of both IFNγ and TNFα have been observed *in vivo* (Lawrence *et al.*, 2016). Both cytokines are potent pro-inflammatory stimuli and as such have the capacity to cause chronic inflammation. Chronic inflammation has been shown to be associated with a number of disease states including certain cancers such as gastrointestinal cancers (Macarthur *et al.*, 2004; Grivennikov and Karin, 2011). Previous studies demonstrated that exposure to TBT at some levels increased the secretion of both TNFα and IFNγ (Hurt *et al.*, 2013; Lawrence *et al.*, 2015). The current study investigated the mechanism of the TBT-induced increases in secretion of both TNFα and IFNγ that were noted in these past studies. This study also addressed whether these TBT-induced increases in TNFα and IFNγ were due simply to release of already existing cytokine, or if TBT was stimulating cellular synthesis of these two cytokines. The fact that levels of TBT that caused increased secretion of these two extremely potent inflammatory signals occur in humans makes examination of the mechanism of this elevation of critical importance.

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Previous studies have shown that TBT is able to activate MAPKs in NK cells (Aluoch and Whalen, 2005; Aluoch *et al.*, 2006; Aluoch *et al.*, 2007). The secretion and production of IFNγ is dependent on mitogen activated protein kinases (MAPKs) signaling (Schoenborn and Wilson, 2007) and in some cases, nuclear factor kappa B (NFκB) (Strengell *et al.*, 2003). TNFα is also regulated by MAPKs and NFκB (Gaestel *et al.*, 2009) as well as TNFα converting enzyme (TACE, also known as ADAM-17) (Goetz *et al.*, 2004). The current studies investigated the role of MAPKs and other signaling pathways, including NFκB and TACE, in TBT-induced increases in IFNγ and TNFα secretion. The results indicated that increased secretion of both IFNγ and TNFα in response to TBT was dependent on the p38 MAPK pathway. The dependence of TBT-induced secretion of IFNγ and TNFα synthesis on p38 activation is consistent with the role of TBT as a cell stressor (Sabio and Davis, 2014). TBT-induced secretion of TNFα was also dependent on TACE activity, but this is true of all TNFα secretion and was not indicative of the mechanism by which TBT was causing increased secretion of TNFα. It has previously been shown that the TBT-induced increases in IL-1β secretion is most significantly dependent on the ERK1/2 MAPK pathway (Brown and Whalen, 2015), in contrast to what was seen with IFNγ and TNFα.

While it was clear that TBT could alter secretion of IFNγ and TNFα, it was not clear if this was due to increased intracellular synthesis of the cytokines or if it was stimulating the release of pre-existing IFNγ and TNFα. The studies presented here indicate that TBT induced small, but consistent, increases in the overall production of both IFNγ and TNFα within 6 hours of exposure to the contaminant and very substantial increases after 24 h. Thus, TBT is causing cells to secrete increased amounts of IFNγ and TNFα, due to its ability to increase the cellular synthesis of each of these cytokines and not simply by triggering the release of already synthesized cytokine. Additionally, we examined whether the p38 MAPK pathway was necessary for the TBT-stimulated increases in IFNγ and TNFα protein synthesis. The data indicated that the p38 pathway was responsible for the increased production of each of these cytokines in response to TBT. This is an important finding, in that it suggests that increased cellular synthesis of these two extremely potent inflammatory proteins is occurring in response to exposure to a ubiquitous environmental contaminant in a p38 dependent manner. Thus, TBT may have the capacity to contribute to a state of chronic inflammation which could lead to increased risk of cancer, certain autoimmune diseases and heart failure (Macarthur *et al.*, 2004; Grivennikov and Karin, 2011; Chowers and Allez, 2010; Shirazi *et al.*, 2017).

Once it was established that TBT was causing an increase in immune cell synthesis of IFNγ and TNFα, it was of interest to examine if this was primarily due to an effect of TBT on the mRNA levels of either or both of these cytokines. It was found that while TBT caused an increase in the levels of both IFNγ and TNFα proteins, increases in the levels of mRNA for either cytokine were not consistent. This indicates that while TBT utilizes the p38 MAPK pathway to elevate the levels of both IFNγ and TNFα in immune cells, it may not necessarily be achieving elevation of these proteins via an increase in the levels of their respective mRNAs. Translational regulation of both IFNγ and TNFα has been described (Mazumder *et al.*, 2010). For instance, TNFα has an AU-rich region (element) (ARE) in the 3’ untranslated region (UTR) of its transcript. A protein designated heterogeneous nuclear ribonucleoprotein-A1 (hnRNP-A1) binds to the ARE in the TNFα transcript and blocks

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translation. This inhibition of TNFα translation can be reversed by a p38 MAPK pathway-dependent phosphorylation of hnRNP-A1 which reduces its ability to bind to the ARE (Buxadé *et al.*, 2005). Future studies will examine whether TBT may have effects on the translational process thus, leading to elevations of these protein levels by that route.

In summary, the current study addresses the mechanism by which TBT leads to increased secretion of both IFNγ and TNFα. The results showed that TBT appeared to predominantly be utilizing the p38 MAPK pathway to cause increased secretion of both cytokines. The question of whether TBT only induced increased secretion of already existing cytokine or whether it induced synthesis of IFNγ and TNFα was also addressed. The data showed that TBT was able to induce increased synthesis of both cytokines within 6 h of exposure at concentrations of TBT that have been found in human blood samples. The increased protein synthesis appears to be dependent on the p38 MAPK pathway but appears to be somewhat independent of increases in the mRNA for either IFNγ or TNFα.

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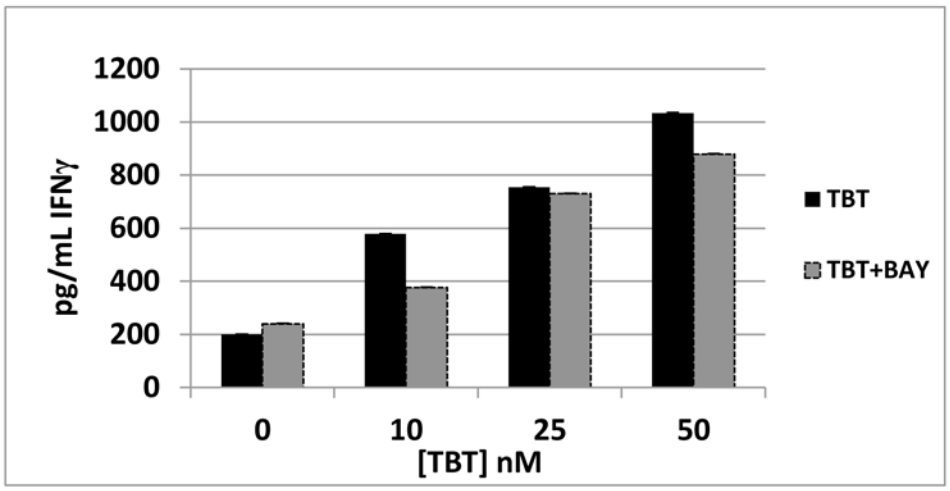

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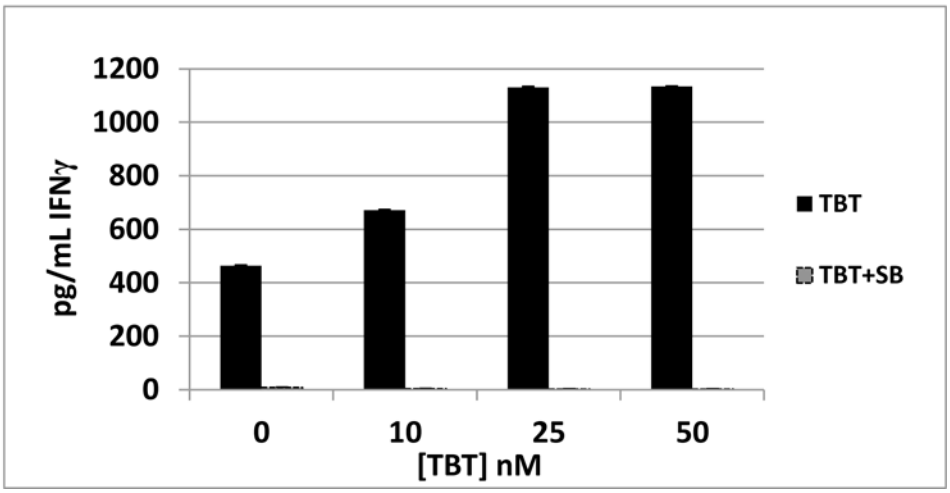

Figure 1.

Effects of pathway inhibitors on secretion of IFNγ from MD-PBMCs exposed to TBT for 24 h. A) Cells were exposed to the NFκB inhibitor (BAY 11–7085) or control for 1 h prior to being exposed to 0, 10, 25, and 50 nM TBT. Results are from a representative experiment (donor F380). These results were reproduced in cells from 3 additional donors. B) Cells were exposed to the p38 inhibitor (SB202190) or control for 1 h prior to being exposed to 0, 10, 25, and 50 nM TBT. Results are from a representative experiment (donor F380). These results were reproduced in cells from 3 additional donors.

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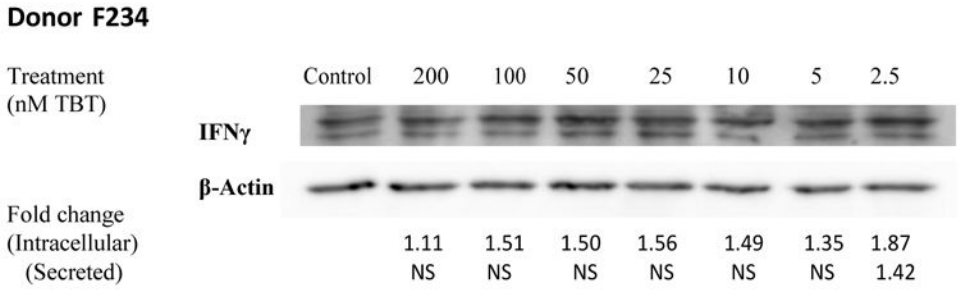
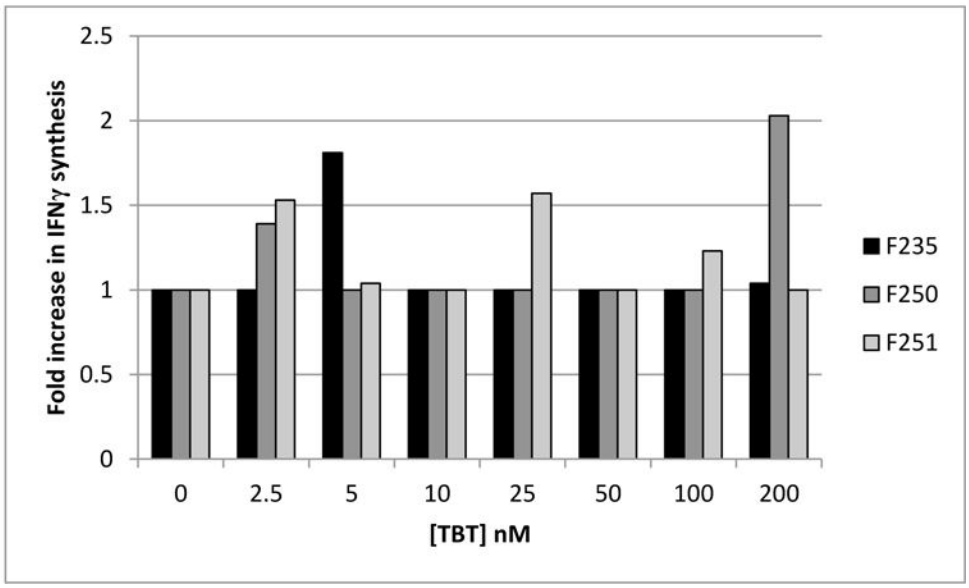
A. 6 hour





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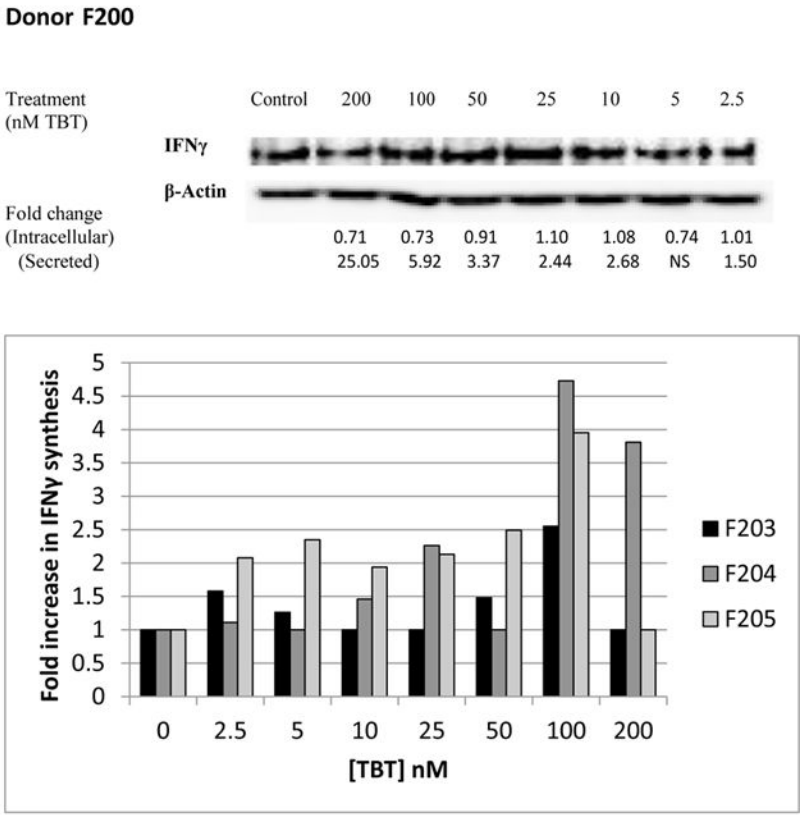
B. 24 hour



C. 24 hour +/- p38 pathway inhibitor

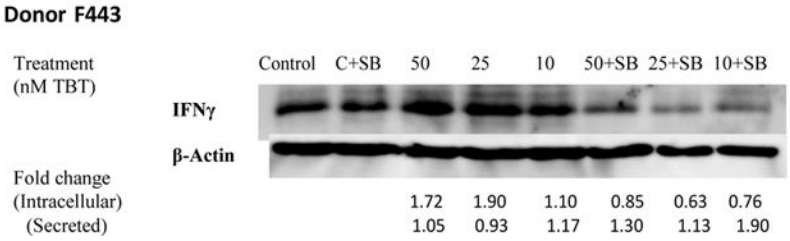


Figure 2.

Effects of varying lengths of exposure to TBT on IFNγ synthesis (secretion + intracellular level) from human MD-PBMCs. A) 6 h exposure to 0–200 nM TBT. The blot with accompanying secretion data is shown for the cells from donor F234. The changes in synthesis (fold increases in secretion+intracellular levels) for cells from 3 other donors are summarized in the bar chart. B) 24 h exposure to 0–200 nM TBT. The blot (with accompanying secretion changes) is shown for cells from donor F200 and the bar chart summarizes the data for 3 other donors. C) 24 h exposure to 0, 10, 25, 50 nM TBT +/− p38

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pathway inhibitor. The blot (with accompanying secretion changes) is shown for cells from donor F443. These results were reproduced in cells from 3 additional donors.

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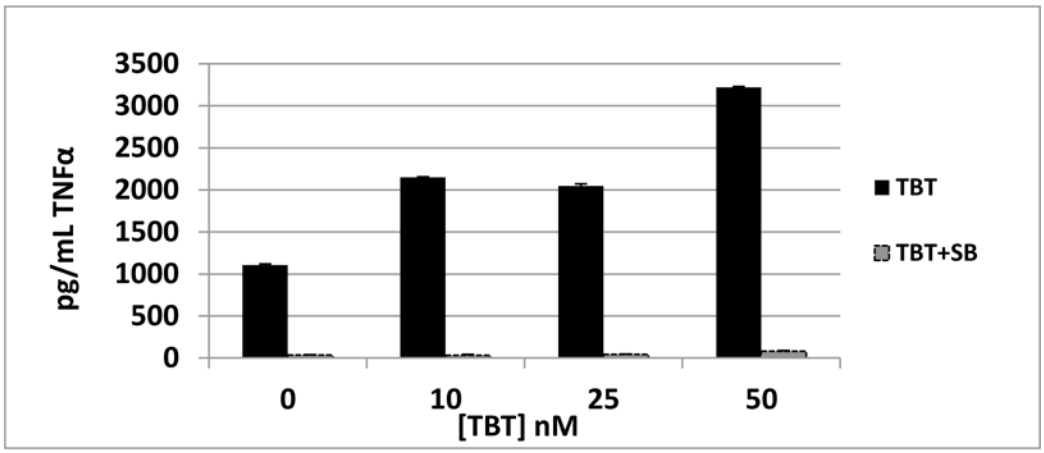
A.



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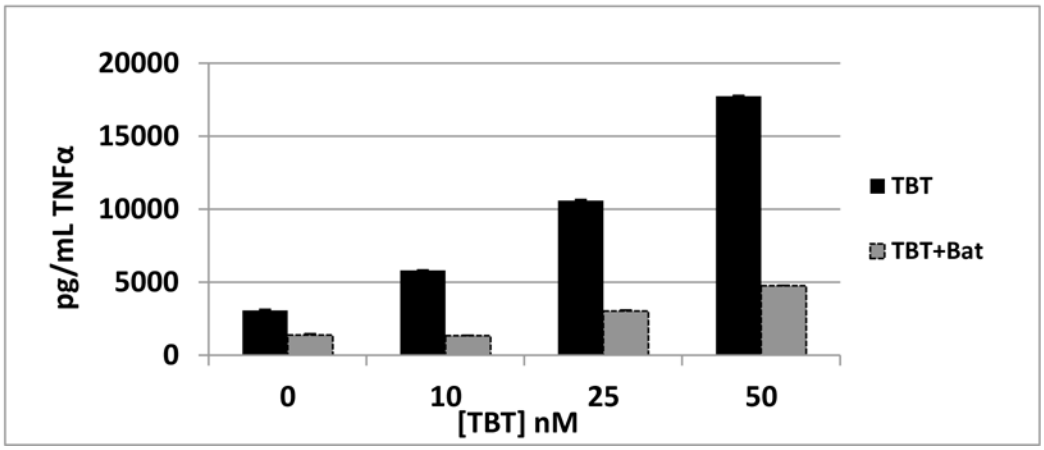

Figure 3.

Effects of pathway inhibitors on secretion of TNFα from MD-PBMCs exposed to TBT for 24 h. A) Cells were exposed to the p38 inhibitor (SB202190) or control for 1 h prior to being exposed to 0, 10, 25, and 50 nM TBT. Results are from a representative experiment (donor F370). These results were reproduced in cells from 3 additional donors. B) Cells were exposed to the TACE inhibitor (Batimastat) or control for 1 h prior to being exposed to 0, 10, 25, and 50 nM TBT. Results are from a representative experiment (donor F272). These results were reproduced in cells from 3 additional donors.

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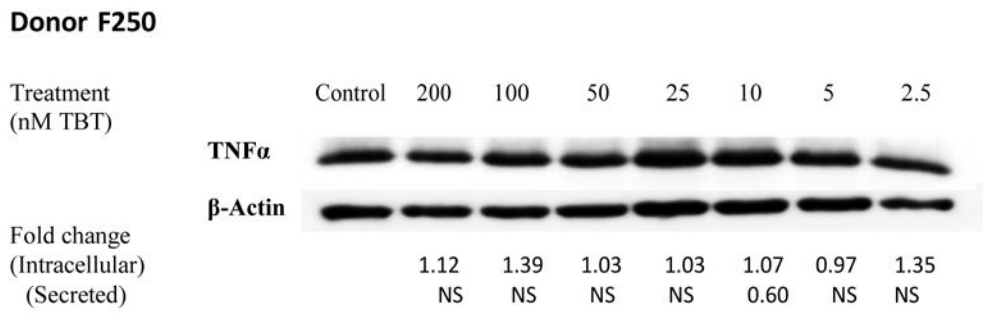
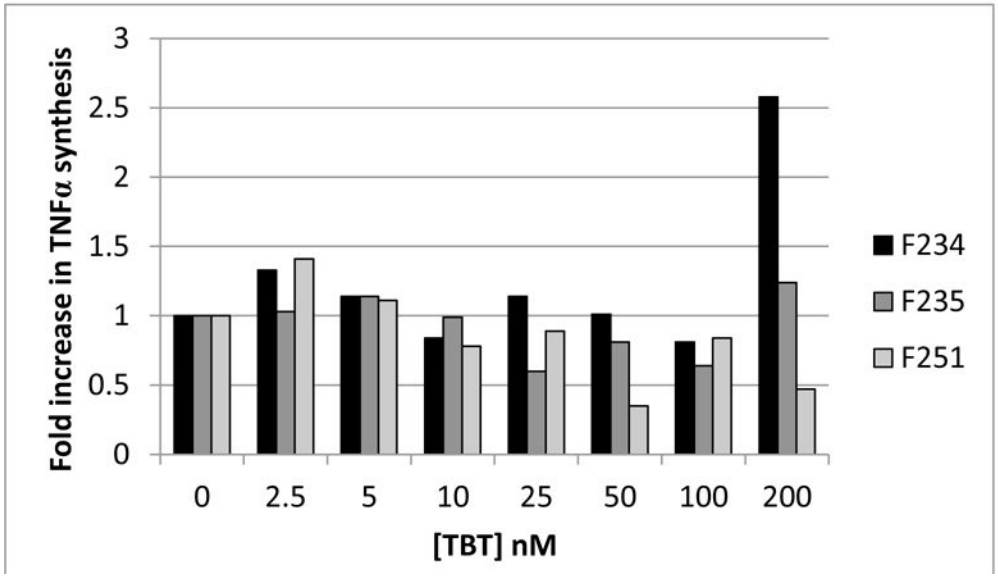
A. 6 hour





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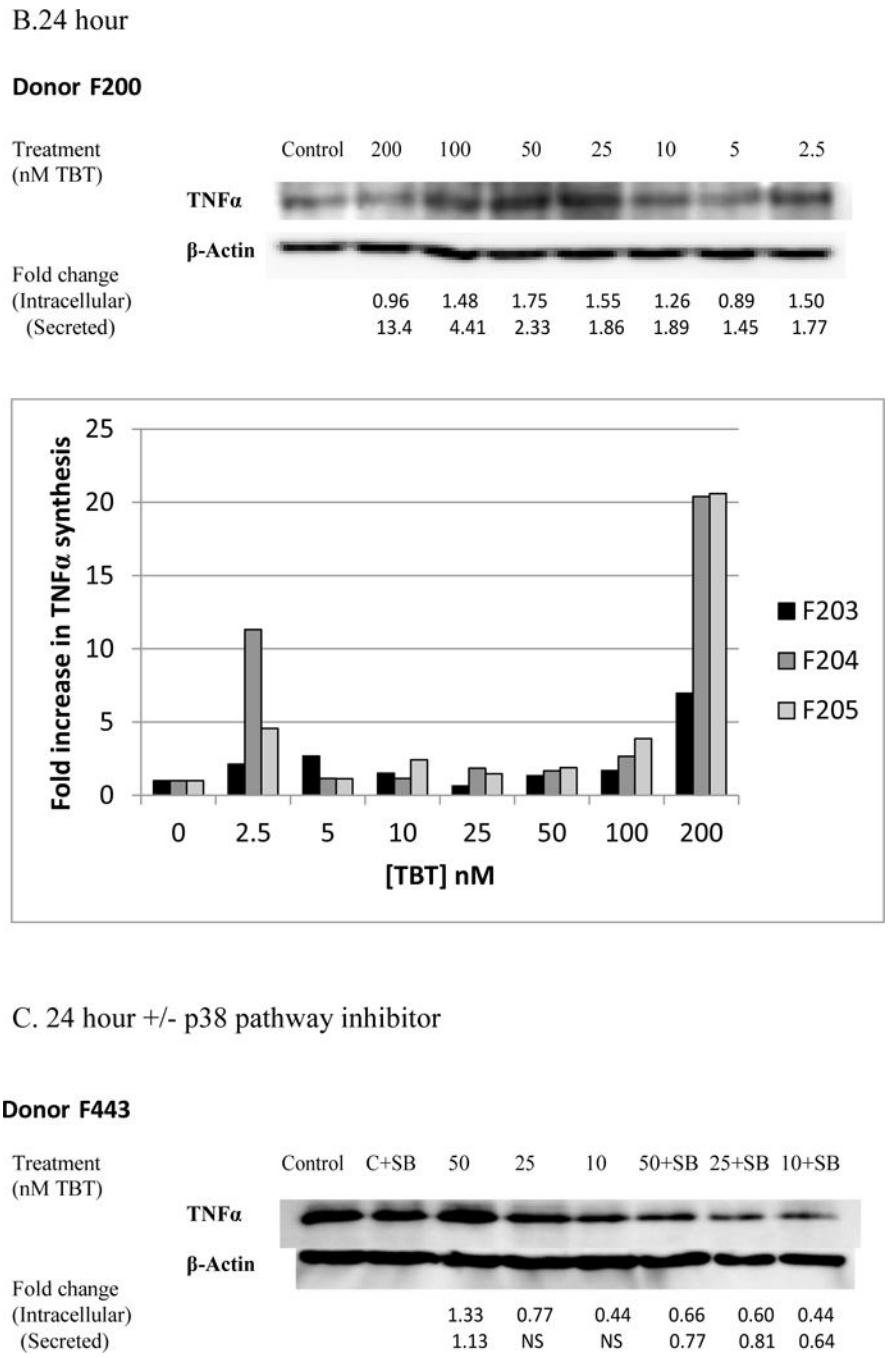

Figure 4.

Effects of varying lengths of exposure to TBT on TNFα synthesis (secretion + intracellular level) from human MD-PBMCs. A) 6 h exposure to 0–200 nM TBT. The blot with accompanying secretion data is shown for the cells from donor F250. The changes in synthesis (secretion+intracellular levels) for cells from 3 other donors are summarized in the bar chart. B) 24 h exposure to 0–200 nM TBT. The blot (with accompanying secretion changes) is shown for cells from donor F200 and the bar chart summarizes the data for 3 other donors. C) 24 h exposure to 0, 10, 25, 50 nM TBT +/− p38 pathway inhibitor. The blot

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(with accompanying secretion changes) is shown for cells from donor F443. These results were reproduced in cells from 3 additional donors.

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Table 1

Effects of 6 h and 24 h exposures to TBT on IFNγ mRNA levels in human MD-PBMCs

6 h	Interferon gamma mRNA in Arbitrary Units (mean±S.D.)			
[TBT] nM	**F546**	**F549**	**F550**	**F552**
0	19.2±0.6	15.4±1.4	26.4±2.4	48.3±8.1
2.5	18.5±0.9	18.4±2.1	21.1±2.0 *	23.4±2.4 *
5	19.9±1.5	19.6±0.8 +	22.4±2.5	28.4±2.1 *
10	15.9±0.2 *	13.6±2.4	17.2±0.3 *	23.6±2.5 *
25	17.1±1.3	15.8±0.4	18.1±0.2 *	21.8±5.1 *
50	12.8±0.2 *	13.4±1.6	16.0±1.0 *	15.6±4.2 *
100	13.6±0.9 *	16.4±1.6	17.0±0.6 *	25.0±2.6 *
200	11.4±0.5 *	17.9±1.5	19.0±1.3 *	22.1±4.1 *

24 h	Interferon gamma mRNA in Arbitrary Units (mean±S.D.)			
[TBT] nM	**F419**	**F420**	**F425**	**F426**
0	36.5±9.1	64.2±1.0	10.1±2.8	24.2±2.9
2.5	66.2±20	64.5±9.3	83.0±35	75.1±19.7
5	33.6±5.5	88.7±18	53.6±9.9 +	86.7±0.4 +
10	29.1±3.6	21.2±4.7 *	30.0±2.8 +	48.8±11.4
25	24.5±5.1	95.5±33	21.1±6.9	28.8±4.0
50	43.9±2.8	104±48	34.9±8.7 +	8.3±4.5
100	30.6±8.7	55.1±1.8	36.0±7.8 +	20.2±2.7
200	18.5±4.6	23.2±1.0 *	15.0±7.3	17.8±6.1

Values are mean±S.D. of triplicate determinations.

+ Indicates a significant increase and

* indicates a significant decrease in mRNA compared to control cells, $p<0.05$

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Table 2

Effects of 6 h and 24 h exposures to TBT on TNFα mRNA levels in human MD-PBMCs

6 h	Tumor necrosis factor alpha mRNA, arbitrary units (mean±S.D.)			
[TBT] nM	F546	F549	F550	F552
0	15.4±1.8	10.8±1.6	22.7±4.3	28.0±1.9
2.5	13.7±0.8	13.0±1.1	22.0±1.0	24.7±1.5
5	15.4±0.6	16.9±1.7 +	24.3±4.1	29±4.1
10	14.8±0.4	14.9±0.4	23.7±2.2	26.9±0.9
25	16.9±0.6	17.0±1.6 +	22.8±1.7	24.1±3.4
50	15.9±0.9	16.0±2.6	19.2±2.3	24.2±4.0
100	20.7±1.2 +	18.5±2.5 +	21.4±0.5	33.9±9.4
200	18.4±0.7 +	15.6±0.4	17.1±3.0	30.4±1.5

24 h	Tumor necrosis factor alpha mRNA, arbitrary units (mean±S.D.)			
[TBT] nM	F419	F420	F425	F426
0	43.6±4.0	26.3±4.6	3.5±1.0	34.7±4.7
2.5	40.4±9.1	24.9±6.5	27.5±2.0 +	32.6±10.1
5	34.0±4.8	17.1±7.0	23.2±4.2 +	21.8±3.9
10	23.0±2.6 *	13.5±6.2 *	11.0±1.4 +	37.5±18.7
25	19.6±6.1 *	15.8±6.8 *	11.7±4.1	28.1±10.7
50	27.4±7.2	21.1±7.6	23.2±6.9 +	25.5±5.9
100	33.0±6.6	16.9±5.2	23.7±6.5 +	24.2±1.0
200	25.2±8.9	15.8±4.1 *	15.0±5.0	26.2±5.9

Values are mean±S.D. of triplicate determinations.

+ indicates a significant increase and

* indicates a significant decrease compared to appropriate control, $p<0.05$